

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph on page 5, starting at line 21 and ending at line 31, with the following new paragraph:

The term “unaltered” as used herein refers to a subunit or monomer that is not fused to a molecule of interest. It is however not limited to the native subunit or monomer; for example it is a recombinant protein wherein one or more amino acids are removed from, replaced in or added to the native subunit. This may be done to modulate the stability and/or the production process, e.g. expression or secretion, of the recombinant protein in a (eukaryotic) host cell. For instance, an unaltered subunit may comprise ~~comprises~~ a subunit provided with a signal peptide or a (SE)KDEL (SEQ. ID. NO.:1) sequence at the C-terminus for retention in the endoplasmic reticulum (ER). The term “unaltered subunit” essentially refers to a native subunit or a slightly modified version thereof wherein the modification does not interfere with multimerization.

Please replace the paragraph on page 5, starting at line 32 and ending at page 6, line 6, with the following new paragraph:

WO-A-9612801 discloses the coordinate expression of an LTA subunit and an LTB subunit fused to a SEKDEL (SEQ. ID. NO.:1) sequence which upon expression together form the CT holotoxin protein complex. Such a protein complex is distinct from a protein complex according to the invention, since it does not comprise ~~an~~ a subunit fused to a molecule of interest according to the present invention. The SEKDEL hexapeptide is not regarded as a molecule of interest. Rather, as noted above, the LTB-SEKDEL (SEQ. ID. NO.:1) subunit is regarded as an “unaltered” subunit. Consequently, according to the terminology of the present invention the complex of WO-A-9612801 is distinct from a protein complex provided herein as it comprises unaltered subunits only.

Please replace the paragraph on page 21, starting at line 18 and ending at page 21, line 28, with the following new paragraph:

Representation of the T-DNA part of the binary vectors pLANTIGEN4, 12, 13 and 15 containing the different LTB and LTB subunit vaccine gene constructs. LB, left T-DNA border sequence; PNOS nopaline synthase promoter; NPTII, neomycin phosphotransferase II gene, selectable kanamycin resistance marker; TNOS, nopaline synthase terminator sequence; PPAT, class I patatin promoter; Gene, cloning site for expression under control of PPAT promoter; RB, right T-DNA border sequence; SP, signal peptide; LT-B, synthetic gene for LTB optimized for expression in plants; KDEL (SEQ. ID. NO.:2), endoplasmic reticulum retention signal; parvo, canine parvo virus (CPV) epitope; Ala, alanine; influenza, HA influenza virus hemagglutinin epitope; CSFV E2, classical swine fever virus E2 glycoprotein lacking transmembrane domain.

Please replace the paragraph on page 25, starting at line 17 and ending on page 25, line 24, with the following new paragraph:

Example 1: Construction of LTB subunit vaccine expression cassettes

A schematic overview of the T-DNA part of the binary plant expression vector pBINPLUS (Van Engelen et al., 1995) and the gene inserts of all the pLANTIGEN vaccine constructs reported here, is represented in Figure 1. All genes were placed under control of the class I patatin promoter (Ppat) for expression in tubers only and in addition harbour a DNA sequence that codes for a KDEL (Lys-Asn-Gln-Leu) sequence (SEQ. ID. NO.:2) at the C-terminus of the respective fusion proteins for retention in the ER (Munro and Pelham, 1987).

Please replace the paragraph on page 25, starting at line 25 and ending on page 25, line 32, with the following new paragraph:

pL4: The design and construction of a synthetic gene for LTB (synLT-B) and the generation of the binary plant expression vector pLANTIGEN4 (pL4) was described before

(Lauterslager et al., 2001). pL4 harbours the synthetic gene for LTB (synLT-B) with a unique BamHI restriction site just after the sequence coding for the mature LTB protein and preceding the sequence coding for KDEL (SEQ. ID. NO.:2). All synthetic sequences were made in such a way and cloned in this unique site, that all were in frame with LTB and the KDEL (SEQ. ID. NO.:2) sequence at the carboxy terminus.

Please replace the paragraph on page 25, starting at line 33 and ending on page 26, line 5, with the following new paragraph:

pL12: The core of the fragment coding for the canine parvo virus (CPV) epitope cloned in pLANTIGEN12 (pL12), codes for the amino acid sequence SDGAVQPDGGQPAVRNERAT (SEQ. ID. NO.:3) (Langeveld et al., 1994). pLANTIGEN12 was made by cloning a synthetic BamHI/BglII fragment coding for the amino-terminal region of the viral protein VP2 of canine parvovirus (CPV) into the unique BamHI site of pL4. The synthetic fragment was made by ligation of fragments derived from oligo's as described before (Florack et al., 1994). Oligo's were from Eurogentec (Belgium).

Please replace the paragraph on page 26, starting at line 6 and ending on page 26, line 12, with the following new paragraph:

pL13: In pLANTIGEN13 (pL13) a fragment coding for the CSFV E2 glycoprotein lacking the C-terminal transmembrane (TM) region was ligated. In wildtype CSFV the E2 glycoprotein is transmembrane bound. pL13 was constructed by cloning a BamHI fragment coding for the E2 mature protein of the classical swine fever virus (CSFV) into the unique BamHI site of pL4. The fragment coding for CSFV E2 was obtained by PCR of pPRb2 (Hulst et al., 1993) using oligos 5'-gttcacacctttcactgaattctgcg-3' (SEQ. ID. NO.:4) and 5'cgcgagaattcagtgaaaaggatgaac-3' (SEQ. ID. NO.:5).

Please replace the paragraph on page 26, starting at line 13 and ending on page 26, line 22, with the following new paragraph:

pL15: In pLANTIGEN15 (pL15), the CPV sequence was cloned twice together with a doubled HA epitope sequence, each separated by two alanine residues for spacing. The HA epitope codes for the decapeptide FERFEIFPKE (SEQ. ID. NO.:6) and represents amino acids 111-120 of PR8 HA-1 (Hackett et al., 1985). CPV is a linear B cell epitope whereas HA is T cell specific. pL15 was constructed by cloning a synthetic fragment coding for a tetrameric sequence consisting of a doubled decapptide of influenza virus hemagglutinin (HA) heavy chain together with a doubled CPV epitope similar to what was cloned in pL12, into the unique BamHI site of pL4. All four epitope sequences were cloned in such a way that they were separated by two alanine residues each.

Please replace the paragraph on page 28, starting at line 19 and ending on page 29, line 27, with the following new paragraph:

Example 4: Chimeric protein complex comprising LTB and LTB-CSFV E2

Binary expression vectors pL4 (Lauterslager et al., 2001) encoding an unaltered subunit and pL13 (LTB-CSFV E2 fusion; for overview see Example 1) encoding a molecule of interest fused to a subunit were introduced in potato by co-transformation using *Agrobacterium tumefaciens* mediated transformation of *Solanum tuberosum* cv. Désirée (De Z.P.C., Leeuwarden, The Netherlands) essentially as described before (Lauterslager et al., 2001) and outlined above (Example 1). To enable co-transformation, prior to infection of stem internodes both recombinant *Agrobacteria* harbouring pL4 and pL13 were mixed in a 1:1 ratio at OD₅₉₅ = 1. The mixed bacterial suspension was used in transformation experiment and regeneration of transformed cells and selection of transgenic shoots was as described before. Seventy-one independent transgenic shoots were obtained and 53 selected and analysed for the presence of pL4 and/or pL13 gene constructs to reveal co-transformed events which were selected for further analysis. To this end, genomic DNA was isolated from leaf

material collected of individual plants by grinding leaf discs of approximately 5mm diameter in 50 μ l urea extraction buffer (62% ureum, 0.5 M NaCl, 70 mM Tris-HCl pH8.0, 30 mM EDTA pH 8.0, 1.5% sarkosyl). An equal volume of phenol/chloroform (1:1) was added and samples were mixed and left at room temperature for 15 min. After mixing, samples were centrifuged for 10 min at 3000 rpm, supernatants were transferred to fresh tubes and 10 μ l 4.4 M ammonium-acetate pH 5.2 was added. To precipitate the genomic DNA 120 μ l isopropanol was added and mixed. Samples were centrifuged for 3 min at 1000 rpm and supernatant removed. The remaining pellets were dried and suspended in water or buffer containing 10 mM Tris-HCl pH8.0 and 1 mM EDTA. Microliter amounts of the genomic DNA samples were submitted to PCR using specific primers that can distinguish pL4 and pL13 gene construct such as but not limited to primers LTB11 (5'-ggtgatcatcacattcaagagcggtgaaacattcaagtc-3') (SEQ. ID. NO.:7) and Tnosminus50 (5'-atgataatcatcgcaagaccg-3') (SEQ. ID. NO.:8). Amplification conditions were: 40 cycles, each 94°C for 30 seconds to enable denaturation, annealing at 56°C for 45 seconds followed by elongation at 68°C for 2 minutes, using AccuTaq polymerase (Sigma-Aldrich) at optimal conditions according to the manufacturer. PCR reaction mixtures were submitted to 1.2% agarose gel electrophoresis in 0.5 X TBE and gels were scanned for the presence of fragments corresponding to the amplified gene constructs of pL4 and/or pL13 for which the predicted sizes are well known and were deduced from their known gene sequences and the use of primers LTB11 and Tnosminus50. Twenty-two plants contained both gene constructs, whereas twenty-eight only contained pL4 and, remarkably, only three had pL13 gene construct. The 22 plants that were positive for both the pL4 and pL13 gene constructs, such as plant number 8, herein further referred to as pL(4+13)8, or plant pL(4+13)16, pL(4+13)31, pL(4+13)39 or pL(4+13)46 were transferred to the greenhouse and grown to maturity for the production of tuber material for further analysis of accumulation of chimeric protein complex.

Please replace the paragraph on page 33, starting at line 22 and ending on page 34, line 6, with the following new paragraph:

For expression in *Escherichia coli* and other prokaryotes the original wildtype *E. coli* sequences for LTB (EtxB) were used. The LTB coding sequence was cloned from pYA3047 [Jagusztyn-Krynicka et al., 1993] which greatly resembles the nucleotide sequence ECELTBP (SWISS_PROT P32890) originally isolated from a porcine *E. coli* strain (Dallas and Falkow, 1980; Leong et al., 1985). A fragment was amplified by PCR using primers LTBbpi (5'-GTGACGAAGACAACATGAATAAAGTAAAATGTTATGTT-3') (SEQ. ID. NO.:9) and LTBbameco (5'-GTGACGAATTCTATGGATCCCCTGGAGCGTAGTTTTTCATACTGATTGCC-3') (SEQ. ID. NO.:10) and a vector comprising wildtype EtxB sequence as template. The resulting BpiI/EcoRI was cloned in a pET21d vector (Novagen) digested with NcoI/EcoRI generating pET-wiLTB1. After verification of nucleotide sequence, the resulting clone was transformed into TOP10F' cells (Invitrogen) for expression studies. An LTB-GFP fusion protein was made by introducing BamHI/BpiI sites at the termini of GFP sequence by PCR using primers GFPbam (5'-GTGACGGATCCGGCTTCCAAGG-3') (SEQ. ID. NO.:11) and LTBGFPbam (5'-GTGACGAAGACAAGATCTTACTTGTACAACATCATCCA-3') (SEQ. ID. NO.:12) and cloned into pET-wiLTB digested with BamHI. After verification of nucleotide sequence, resulting clone pET-wiLTB-GFP2 was transformed into TOP10F' cells for expression studies (Example 8).

Please replace the paragraph on page 36, starting at line 31 and ending on page 37, line 22, with the following new paragraph:

Example 11. Chimeric complex of LTB and LTB-VHSV G

A genetic fusion of LTB and the spike glycoprotein G from viral hemorrhagic septicemia virus similar to sequence X66134 (EMBL) and published by Lorenzen et al. (Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus, and immunization of

rainbow trout with the recombinant protein. (J. Gen. Virol. 74 (1993) 623-630) is made as follows: a unique BamHI site is introduced by PCR amplification of VHSV G sequence and primers VHSVGSmaI (5'-gatcgacccgggagatctaagtcacagaccgtctgacttctggagaactgc-3') (SEQ. ID. NO.:13) and VHSVGBamHI (5'-tctggtgatccgcagatcactcaacgacctccgg-3') (SEQ. ID. NO.:14). PCR conditions are 30 sec. at 96°C, 30 sec. 60°C and 45 sec. at 72°C for 30 cycles using Pwo polymerase. The resulting fragment is excised with BamHI and SmaI and cloned in frame in unique BamHI site of pLANTIGEN4 harbouring the synthetic gene for LTB. The resulting gene sequence under control of patatin promoter and nopaline synthase terminator sequence is named pLANTIGEN24 (pL24). A co-transformation of potato is performed with pL4 and pL24 generating numerous pL(4+24) plants. The presence of pL4 and/or pL24 gene constructs is confirmed by PCR as described before. Plants that are positive for both gene constructs are allowed to form tubers. Tubers are harvested and analysed for GM1-binding complexes using GM1-ELISA. The presence of VHSV G protein in complexes is confirmed by incubation with monoclonal antibodies IP1H3, 3F1H10 and 3F1A2 (Lorenzen et al, 2000. Three monoclonal antibodies to the VHS virus glycoprotein: comparison of reactivity in relation to differences in immunoglobulin variable domain gene sequences. Fish & Shellfish immunology 10: 129-142). Chimeric complexes can further be characterized by Western blotting of tuber extracts run on SDS-PAGE under semi-native conditions and using the anti LTB5 mAb VD12 and 1P1H3, 3F1H10 and 3F1A2.

Please replace the paragraph on page 37, starting at line 24 and ending on page 38, line 5, with the following new paragraph:

Example 12. Chimeric complex of LTB and LTB-SVCV G

A BamHI/BglII fragment comprising the complete SVCV G gene of spring viremia of carp virus (Genbank accession nr. NC002803) and for making a genetic fusion with LTB, was amplified using oligonucleotides SVCVG1 (5'-tctggtctcgagatcccatattgttccatc-3') (SEQ. ID. NO.:15) and SVCVG2 (5'-gatcgaggatccaagtcacaaactaaagaccgcatttcg-3') (SEQ. ID. NO.:16). The resulting fragment was excised with BamHI and XhoI and cloned in the BamHI/XhoI site of

pL4 coding for LTB;p thereby generating pLANTIGEN27 (pL27). The resulting gene placed under control of the tuber specific patatin promoter (pLANTIGEN27) was introduced in *A.tumfaciens* for transformation of potato. A co-transformation of pL4 and pL27 was performed and transgenic plants were evaluated for the presence of both gene constructs by PCR as described. Transgenic plants harbouring pL(4+27) gene constructs were selected and grown to maturity in the greenhouse. Tubers were analysed for accumulation of GM1-binding complexes by GM1-ELISA and for the presence of SVCV G protein using specific mAbs. The subunit composition of the protein complexes was visualized by Western blotting after semi-native SDS-PAGE as described.

Please replace the paragraph on page 38, starting at line 8 and ending on page 38, line 24, with the following new paragraph:

Example 13. Chimeric complex LTB and LTB-ClyIIA

Actinobacillus pleuropneumoniae serotype 9, reference strain CVI13261, is grown on heart infusion agar (Difco) containing 0.1% V-factor (NAD). High molecular weight DNA is isolated by proteinase K/SDS lysis, followed by phenol/chloroform extraction and precipitation of resulting genomic DNA. The ClyIIA gene from *Actinobacillus pleuropneumoniae* serotype 9 (GenBank-EMBL accession nr. X61111) is cloned from genomic DNA isolated of serotype 9 strain by PCR using oligonucleotides Cytol1 (5'-gatccatggcaaaaatcactttgtcatc-3') (SEQ. ID. NO.:17) and Cytol4 (5'- atcggatccctattaagcggctctagctaattg-3') (SEQ. ID. NO.:18). Subsequently, a BamHI site is also introduced at the amino terminus of the ClyIIA gene by PCR and the resulting fragment excised with BamHI was cloned in pET-wiLTB as described for expression in *E.coli* and generating pET-wiLTB-ClyIIA. Chimeric complexes can be obtained by co-expression of pET-wiLTB and pET-wiLTB-ClyIIA upon induction with IPTG. Alternatively, inclusion bodies obtained upon overexpression of pET-wiLTB-ClyIIA in *E.coli* and purified recLTB are mixed and solubilized by 8M Urea and dialysed against Tris buffer as described to renature pentameric complexes.